Stilbenoid Prenylation Pathway Discovery in Peanut using Targeted Transcriptomics

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Abstract

In peanut, *Arachis hypogaea*, defense responses to biotic and abiotic stresses include the synthesis of prenylated stilbenoids, with over 45 such compounds identified to date. The diversification of secondary metabolites in plants is expanded by prenylation activities, and in recent studies this modification has been shown to enhance biological activities of polyphenolic compounds.

We describe our discovery of genes responsible for stilbenoid prenylation[1] as well as studies underway to understand the regulation of these metabolic programs in peanut. Sequencing RNA from a well-characterized peanut hairy root system, we built a transcriptome reference and correlated transcripts with metabolites produced over a time course of elicitation. Transcripts encoding candidate enzymes were identified and characterized functionally by heterologous transient expression. Prenyltransferases we call AhR4DT-1 and AhR3'DT-1 catalyze distinct reactions, and our studies suggest that these act in the first committed steps that convert non-prenylated into prenylated stilbenoids.

Here we highlight the functional genomics analyses that led to these discoveries, and our ongoing approaches to find other genes that act in the regulation of this defensive metabolic program. Identification of the first plant stilbenoid-specific prenyltransferases here advances our understanding of this specialized gene family, and contributes some of the functional definition that is needed generally to refine the annotations of plant genomes.

Experiment Summary

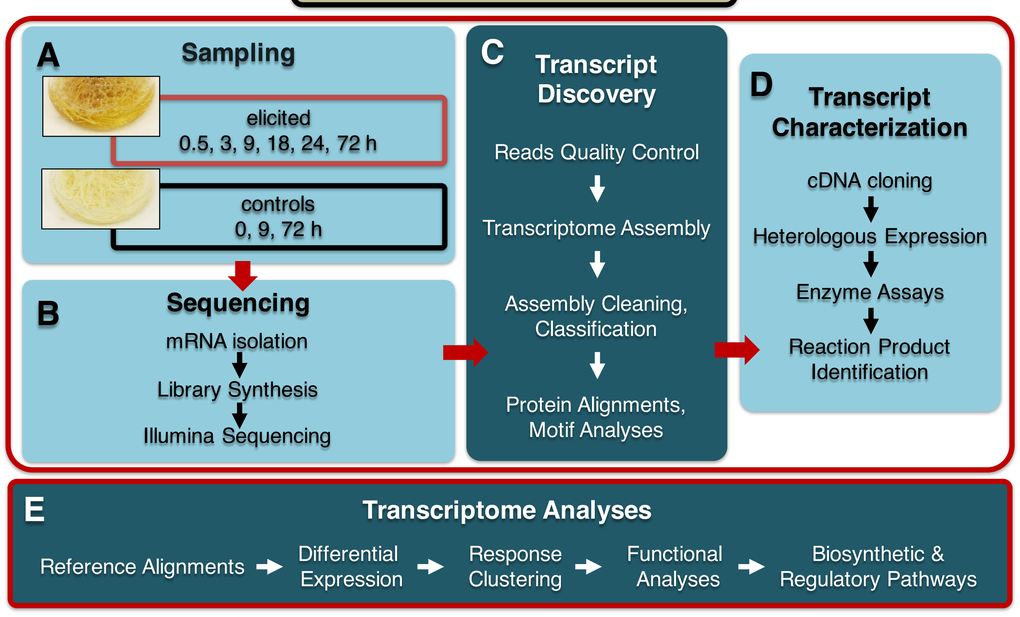

Figure 2. Workflow of RNASeq, Gene Discovery and Characterization (*light*, laboratory; *dark*, computing). **A,** Peanut hairy root cultures were treated with MeJA + CD (elicited) or media alone (control). **B,** Total RNA was extracted, mRNA enriched by affinity to poly-dT, and stranded TruSeq (Illumina) libraries were prepared and sequenced as paired reads, an average of 52.5 M per sample. **C,** Bioinformatics included reads quality assessment and trimming, use of four complementary assembly software approaches varying kmers[7], and transcript quality assessment and classification using software of the EviGenes suite[8]. Translated transcripts were aligned initially to known flavonoid prenyltransferases[9] to find stilbenoid prenyltransferase candidates, then reduced to those with enzymatic characteristics determined previously[6]. **D**, Candidates were cloned and expressed as cDNAs in a heterologous plant system to test for activities. Reactions products were analyses by both HPLC, 1-D and 2-D NMR[6]. **E**, All transcriptome data were subjected to further analyses to determine differential expression over the experimental time course, to group those showing similar patterns of transcript accumulation during elicitation, and to attribute proteins to known biochemical pathways.

Results: Metabolic Gene Discovery

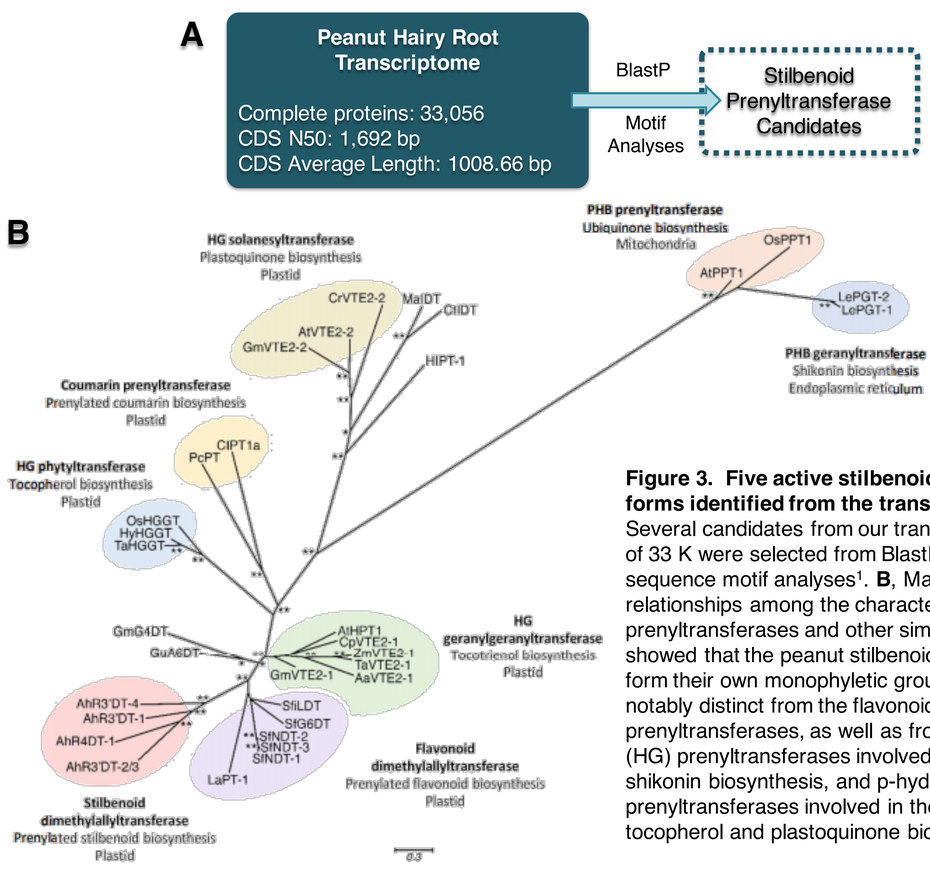

Figure 3. Five active stilbenoid prenyltransferase forms identified from the transcript reference. A, Several candidates from our transcriptome reference of 33 K were selected from BlastP results after further sequence motif analyses[1]. **B**, Maximum likelihood relationships among the characterized prenyltransferases and other similar enzymes showed that the peanut stilbenoid prenyltransferases form their own monophyletic group. This clade is notably distinct from the flavonoid and coumarin prenyltransferases, as well as from homogentisate (HG) prenyltransferases involved in ubiquinone and shikonin biosynthesis, and p-hydroxybenzoate (PHB) prenyltransferases involved in the tocotrienol, tocopherol and plastoquinone biosynthetic pathways.

The Experimental System

Just as peanut plants in the field produce and secrete diverse prenylated stilbenoids as antibiotics when invaded by fungal pathogens[2], hairy root cultures of peanut produce these compounds when stress-signaling is mimicked by the addition of methyl jasmonate (MeJA), hydrogen peroxide or sodium acetate. The hairy root culture system was developed[3] and optimized[4,5,6] for for use in targeted stilbenoid production and transcript association studies described here.

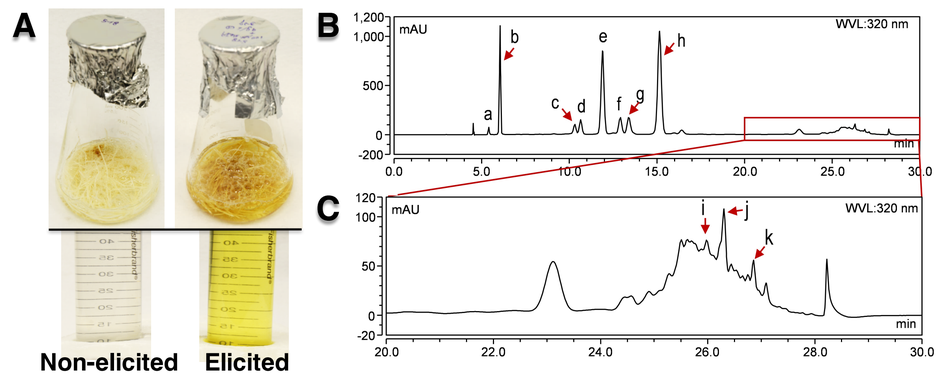

Figure 1. Stilbenoid production in a peanut hairy root system. A, A mixture of stilbenoid monomers and dimers are secreted from peanut hairy root cultures after elicitor treatment of MeJA + methyl-β-cyclodextrin (CD). **B,** HPLC chromatogram of ethyl acetate extract from the elicited culture medium. Stilbenoids: **a**, piceatannol; **b**, resveratrol; **c**, arachidin-5; **d**, arachidin-5 derivative; **e**, arachidin-1; **f**, arachidin-2; **g**, arachidin-2 derivative; **h**, arachidin-3. **C,** Close-up of 20-30 min. shows highly non-polar prenylated stilbenoids and derivatives including **i-k**, dimers.

Results: Gene Discovery Validation

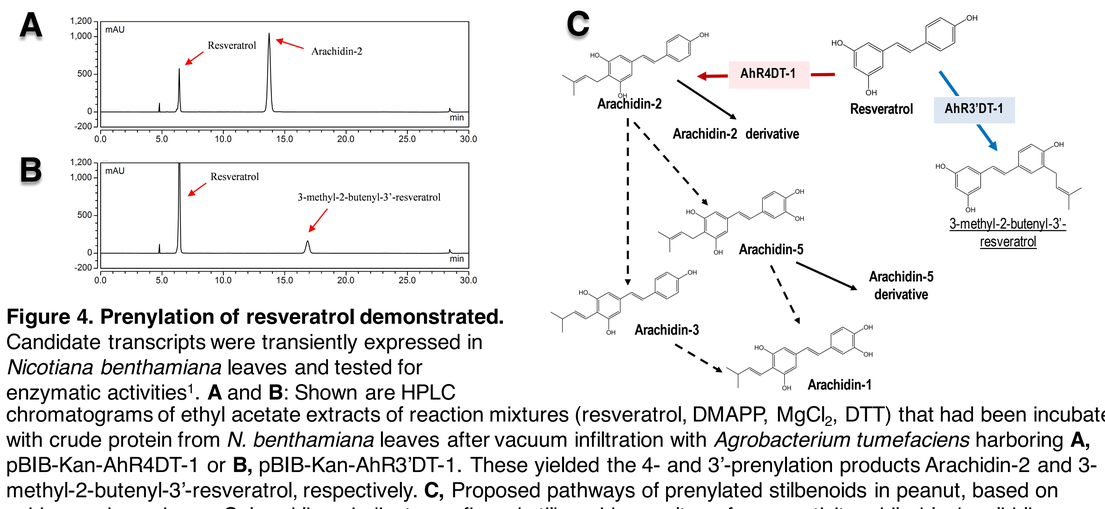

Figure 4. Prenylation of resveratrol demonstrated. Candidate transcripts were transiently expressed in *Nicotiana benthamiana* leaves and tested for enzymatic activities[1]. **A** and **B**: Shown are HPLC chromatograms of ethyl acetate extracts of reaction mixtures (resveratrol, DMAPP, $MgCl_2$, DTT) that had been incubated with crude protein from *N. benthamiana* leaves after vacuum infiltration with *Agrobacterium tumefaciens* harboring **A,** pBIB-Kan-AhR4DT-1 or **B,** pBIB-Kan-AhR3'DT-1. These yielded the 4- and 3'-prenylation products Arachidin-2 and 3-methyl-2-butenyl-3'-resveratrol, respectively. **C,** Proposed pathways of prenylated stilbenoids in peanut, based on evidence shown here. Colored lines indicate confirmed stilbenoid prenyltransferase activity, while black solid lines indicate other confirmed reactions. Proposed reactions are shown as dashed black lines. The underlined product of AhR3'DT-1 has not been identified in peanut. Not shown are activites of additional candidates and characterized activities of AhR4DT-1 and AhR3'DT-1 on stilbenoid substrates other than resveratrol[1].

Results: Biosynthesis and Regulatory Response Clusters

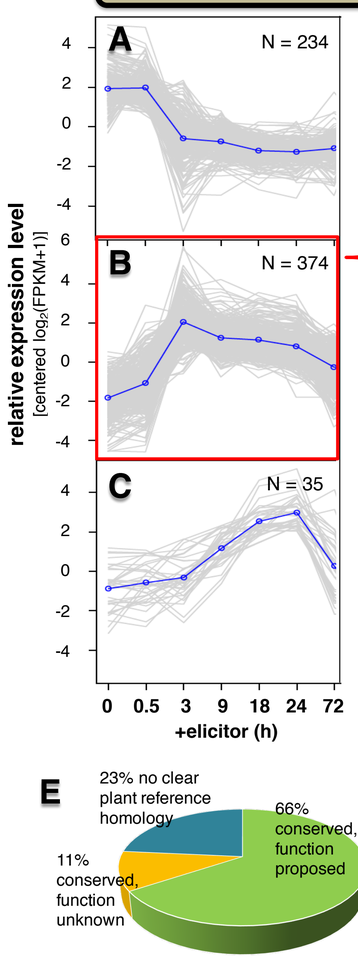

Figure 5. Defining metabolic conservation and novelties to understand system regulation. We are dissecting the entire transcriptome to identify candidate regulators of stilbenoid production and modification. Alignments to diploid *A. duranensis* and *A. ipanensis* genomes[10], and to a concatenated pseudotetraploid reference, confirmed homeologous and allelic transcripts we had defined[6]. RNAseq reads of each sample were mapped and comparatively quantified. Transcripts differentially expressed ($p<0.001$) during elicitor treatment were clustered according to accumulation patterns. *Left*, shown are three transcript clusters exhibiting elicitation responses: **A**, downregulation; **B**, early upregulation; **C**, late upregulation. KEGG[11] ontologies encoded by all transcripts were mapped in iPATHv2. *AhR4DT-1* (Figs. 3 and 4) falls into Cluster B, where phenylpropanoid biosynthesis ontologies were notably enriched. **D**, highlighted in **red** are phenylpropanoid pathway members of Cluster B. In pink are those present in the system whose accumulation did not vary over the 72 h elicitation. 8.3% of protein-coding transcripts were unique sequence discoveries in our experiments (not annotated in *Arachis* diploids), a mix of genes uniquely active in tetraploid *A. hypogea* and genes uniquely active in our elicitation experiments. **E**, Considering elicitation-responsive genes, 11% of orthologs have gene model annotations with functions currently 'unknown' in other plant references.

Summary

◆ A transcript reference of 33 K complete protein coding sequences was built from RNA sequencing of a metabolically well-defined peanut hairy root culture system undergoing defense response.

◆ Stilbenoid prenyltransferase sequences were discovered for the first time. Their activities toward stilbenoid substrates such as resveratrol were validated *in vivo*.

◆ Bioinformatic analyses of this transcriptome reference as a whole is leading to greater definition of the components of stilbenoid production and modification in plants.

References:
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